*THE CROSS-LINKING OF COLLAGEN AND ELASTIN: ENZYMATIC CONVERSION OF LYSINE IN PEPTIDE LINKAGE TO α-AMINOADIPIC-δ-SEMIALDEHYDE (ALLYSINE) BY AN EXTRACT FROM BONE**

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Current evidence indicates that the initial step in the cross-linking of both collagen[1] and elastin[2, 3] is the conversion of peptide-bound lysine to α-aminoadipic-δ-semialdehyde. For the sake of brevity, we have given the latter compound the trivial name allysine to indicate that it is an aldehyde derived from lysine. In the case of collagen, one lysyl residue per chain of $\sim 10^3$ amino acids[4] has been found to be converted to this aldehyde as compared to 5–16 lysyl residues per 10^3 amino acids in elastin.[2, 5] The mechanism of formation of the actual cross-links is probably due both to the spontaneous condensation of aldehydes and to their reaction with still other groups on these proteins.[3, 6]

The enzyme converting lysyl residues to allysyl residues has not been previously identified. In this paper, we will describe assays for, and some of the properties of, an enzyme from bone that converts peptide-bound lysine to allysine. The enzyme is inhibited by physiologically active levels of the lathyrogen β-aminopropionitrile, a compound previously shown *in vivo* and in tissue culture to inhibit the cross-linking of collagen[7, 8] and elastin[9] by blocking the lysine-to-allysine conversion.[2, 4]

Materials and Methods.—Preparation of substrate: Eighteen aortas from 16-day-old embryonic chicks were incubated in a 50-ml Erlenmeyer flask with 10 ml of Eagle's minimal essential medium lacking free lysine but containing 20 μg/ml β-aminopropionitrile fumarate (BAPN) and either DL-lysine-6-^{3}H (200 μc) or L-lysine-U-^{14}C (50 μc). After being flushed with a mixture of 95% O_2 and 5% CO_2, the stoppered flasks were incubated with shaking for 24 hr at 37°. Following incubation, the aortas were rinsed with distilled water and lyophilized. Lyophilized aortas were ground in a glass homogenizer at room temperature in 0.16 *M* saline, and the homogenate was centrifuged at 11,000 *g*. The supernatant was discarded and the pellet was rehomogenized in saline and reisolated by centrifugation twice more. In general, substrate equivalent to the pellet from two aortas containing approximately 500,000 cpm ^{3}H or 100,000 cpm ^{14}C was used per incubation.

Preparation of bone extract (105S): Tibias and femurs from 16-day-old embryonic chicks were homogenized (6 bones/ml) in 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4) in a Polytron homogenizer. The supernatant (105*S*) obtained by centrifuging the homogenate (105,000 *g* for 2 hr at 5°) was used as the source of the enzyme and contained 5–8 mg protein/ml.

In some experiments, 105*S* extracts were incubated at 23° with and without 50 μg/ml of BAPN for 2 hr. These extracts were then dialyzed extensively against 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4) at 5°.

Standard incubation mixture: The standard incubation mixture consisted of the labeled substrate and 1.5 ml of 105*S*. Since allysine may be an intermediate in the degradation of free lysine in mitochondria,[10] sufficient carrier lysine (1 mM) was added to the reaction mixture to dilute any free lysine-6-^{3}H carried over in the preparation of the labeled substrate. BAPN (1–50 μg/ml) was added to some of the tubes, and the final volume was

adjusted to 1.7 ml with 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4). Toluene (0.1 ml) was added to inhibit the growth of bacteria, and in general the incubation was carried out for 8 hr in stoppered test tubes at 37° with shaking. Since little activity was noted below 23°, the reaction was stopped by freezing the samples.

Assay with lysine-6-^{3}H-labeled substrate: Following incubation, the water in the sample was isolated by distillation,[11] and 1 ml was counted in Bray's solution in a liquid scintillation counter.

Assay with lysine-^{14}C-labeled substrate: Following incubation, the pellets were reisolated by centrifugation (105,000 *g* for 1 hr at 5°) and oxidized with performic acid to convert allysine-^{14}C to α-aminoadipic acid-^{14}C (Aad-^{14}C).[2] After hydrolysis for 72 hr in 6 *N* HCl at 107°, the amino acids were separated on an automatic amino acid analyzer,[12] and the radioactivity in the column effluent was monitored continuously.[13]

Results.—To survey tissue extracts for their ability to convert lysyl residues to allysine, a tritium-release assay, patterned after that used in the study of protocollagen hydroxylase,[11] was developed. Elastin was chosen as the substrate because more allysine is formed during the cross-linking of elastin than collagen. Aorta was biosynthetically labeled with lysine-6-^{3}H in the presence of BAPN to inhibit prior deamination, and an insoluble elastin-containing fraction of the aorta was prepared. The assay for the detection of allysine formation is based upon the enzyme-dependent release of tritium from the lysine-6-^{3}H-labeled protein substrate while lysyl residues are converted to peptide-bound allysine (Fig. 1).

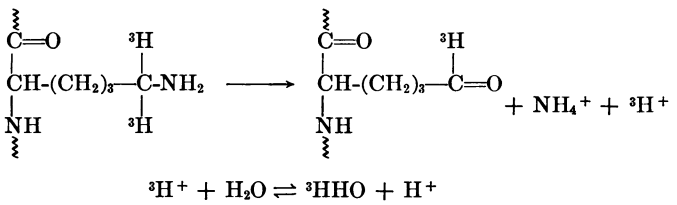

FIG. 1.—Release of tritium during allysine formation.

The tritiated water isolated by distillation[11] serves as a measure of the extent of the reaction.

Although enzymatic activity has been detected in 105*S* from embryonic chick skin and aorta, bone has proved to be the largest and most reproducible source of the enzyme. The studies reported here were all carried out with extracts from bone. When 105*S* was incubated with lysine-6-^{3}H-labeled substrate, tritium was released in a linear fashion for approximately eight hours (Fig. 2). BAPN (50 μg/ml) markedly inhibited tritium release. Since BAPN prevents the formation of allysine in collagen and elastin *in vivo*, the difference between tritium release in the presence and absence of BAPN would be expected to be a measure of the formation of the aldehyde. The relatively slow course of tritium release may be due to the fact that the substrate is insoluble and not readily accessible to the enzyme. About 0.6 per cent of the total tritium in the substrate had been released when the reaction was complete.

Levels of BAPN of 1 μg/ml inhibited tritium release 50 per cent, and 5 μg/ml inhibited it maximally (Fig. 3). Similar concentrations of BAPN were previously found to inhibit the formation of the desmosine cross-links in embryonic chick aorta[9] and the cross-linking of collagen in embryonic chick bone in tissue

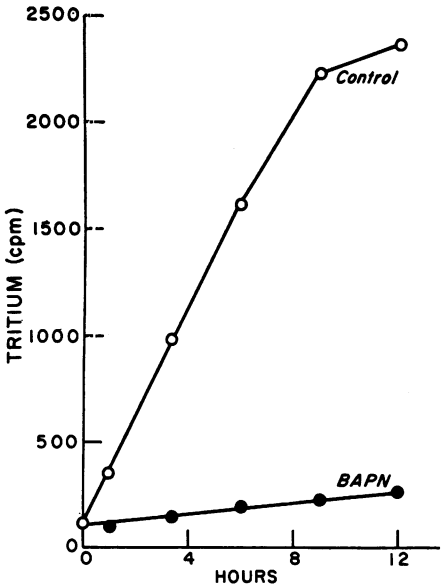

FIG. 2.—Release of tritium from lysine-6-^{3}H-labeled substrate by an extract from bone.

culture.[14] Equimolar concentrations of fumarate did not inhibit tritium release.

To demonstrate that the expected product, allysine, was generated by the enzyme, its hydrolysis-stable oxidation product, α-aminoadipic acid (Aad), was measured by using lysine-^{14}C-labeled substrate in the incubation mixture. After hydrolysis and oxidation, Aad-^{14}C was separated on an automatic amino acid analyzer and measured by continuous monitoring of the radioactivity in the column effluent. The radioactivity in typical chromatograms is shown in Figure 4 for substrate incubated with enzyme, with and without BAPN (50 μg/ml). The chromatograms are identical with the exception that much less Aad-^{14}C was found in the system inhibited by BAPN. Some radioactivity was found to co-chromatograph with aspartic acid, glutamic acid, and proline. This pattern of radioactivity was found in hydrolysates of unincubated substrate and probably arose from the metabolic degradation of free lysine-^{14}C and the reutilization of its carbon. The pattern was not altered during incubation with enzyme.

Comparisons of tritium release and allysine-^{14}C (measured as Aad-^{14}C) formation are shown in Table 1. BAPN inhibited activity in both assays to a similar extent. Substrate incubated without added enzyme released more tritium than did the BAPN-inhibited samples. It seems likely that this activity is due to enzyme present in the aorta that was carried along in the preparation of the substrate, since this activity was also inhibited by BAPN and was absent in boiled samples. Boiled enzyme showed no activity beyond the inherent substrate activity.

Some difference between the two assays might be expected. While all the tritium released as a result of the conversion of lysyl residues to allysyl residues should be measured in the tritium-release assay, some of the allysine-^{14}C formed might react to form compounds not detected by oxidation to Aad-^{14}C. However, in general, the two assays have given similar results.

To show that the enzyme was acting on lysine in peptide linkage, the enzyme was incubated with free DL-lysine-6-^{3}H and L-lysine-^{14}C. No enzymatically dependent release of tritium or formation of Aad-^{14}C was observed. In addition, enzymatically dependent tritium release from lysine-6-^{3}H-labeled substrate was not altered by the presence of 10 mM free lysine. Furthermore, aortas incubated with DL-lysine-6-^{3}H in the presence of cycloheximide (25 μg/ml), which reduced the incorporation of tritium into the substrate by 95 per cent, failed to synthesize detectable substrate.

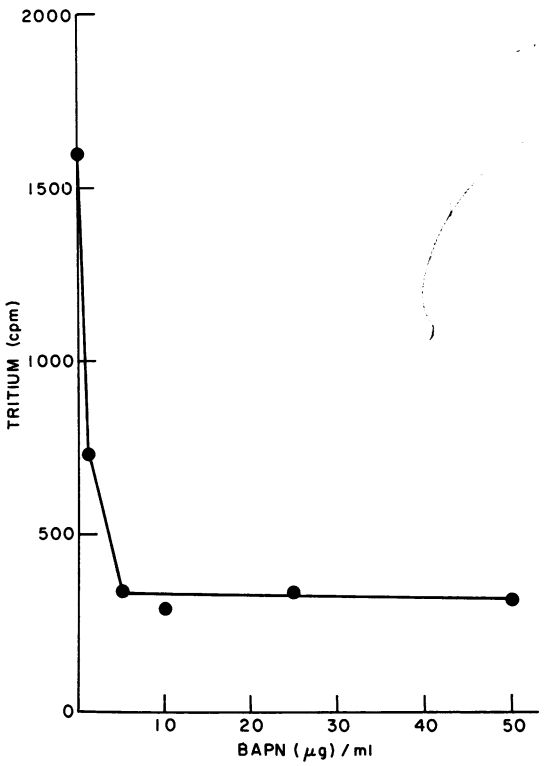

FIG. 3.—Enzymatic release of tritium from lysine-6-^{3}H-labeled substrate. Effect of varying concentrations of β-aminopropionitrile (BAPN).

The radioactivity in the DL-lysine-6-^{3}H as received from the manufacturer was found by amino acid analysis to be entirely associated with lysine. However, previous studies have shown that in DL-lysine-6-^{3}H prepared by the catalytic hydrogenation of 5-cyano-α-aminopentanoic acid, a portion of the tritium (~26%) is in the C-5 position,[15] a fact which suggests that tritium release might be due in part to the formation of hydroxylysine. This was excluded as follows: Protocollagen hydroxylase requires the readily dissociable cofactors Fe^{+2}, ascorbic acid, and α-ketoglutarate and is inactivated by dialysis.[16] The enzyme described here was fully active after dialysis against 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4) for 24 hours, and addition of Fe^{+2}, ascorbic acid, and α-ketoglutarate failed to enhance tritium release. Although β-aminopropionitrile fumarate (50 μg/ml) caused a 20 per cent inhibition of protocollagen hydroxylase, an equimolar concentration of fumarate alone had a similar effect, which suggests that the effect was due to fumarate. (We are indebted to Dr. John Gribble for these determinations.) As already noted, fumarate alone did not inhibit the enzyme responsible for allysine formation.

To determine whether BAPN would inactivate *in vitro* the enzyme described here, control 105*S* extract was incubated with and without 50 μg/ml BAPN for two hours at 23°. Subsequently, the extracts were dialyzed extensively to remove free BAPN. Extracts incubated with BAPN had markedly less activity than did extracts incubated without BAPN (Fig. 5). This decrease in activity

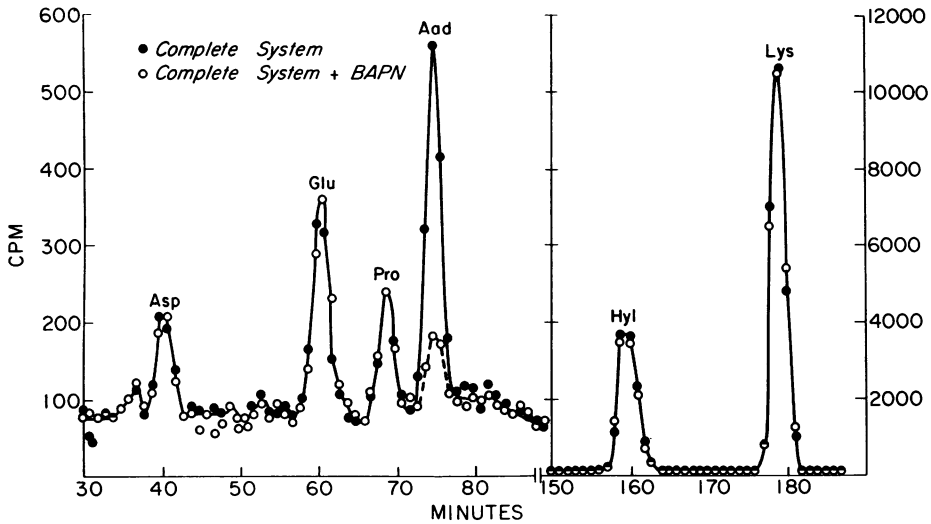

FIG. 4.—Distribution of radioactivity (^{14}C) in a typical amino acid chromatogram after lysine-^{14}C-labeled substrate was incubated with enzyme.

was not due to free BAPN that was not removed during dialysis since a mixture of control and BAPN-incubated 105*S* extracts was as active as an equal amount of control 105*S* extract alone.

Discussion.—An enzyme capable of converting lysine in peptide linkage to allysine has been detected in extracts from embryonic chick bone. Previous studies have shown this reaction to be an early and essential step in the cross-linking of collagen and elastin. The lathyrogen BAPN, a compound known to block the cross-linking of collagen and elastin at this step, inhibits the enzyme at physiologically active levels. Presumably inactivation of this enzyme represents the primary lesion in lathyrism.

The insoluble aortic substrate used here is not well defined but would be expected to contain all the collagen and elastin present in the homogenate. To function as substrate, lysine must be incorporated into peptide linkage. Free lysine is not a substrate for the enzyme, and labeled substrate does not accumulate in aortas incubated with cycloheximide.

TABLE 1. *Comparison of tritium release and allysine-^{14}C formation.**

Addition to prelabeled substrate	Tritium Release		Aad-^{14}C Formation	
	Cpm	Per cent	Cpm	Per cent
(1) Enzyme	1593	100	818	100
(2) Enzyme + BAPN (50 μg/ml)	313	20	147	18
(3) Enzyme replaced by 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4)	666	42	306	38
(4) Enzyme replaced by 0.16 *M* NaCl, 0.1 *M* phosphate buffer (pH 7.4) + BAPN (50 μg/ml)	246	15	278	34
(5) Enzyme replaced by boiled enzyme	699	44	216	26
(6) Enzyme incubated at 5°	93	6	120	15

* Allysine-^{14}C is measured as Aad-^{14}C.

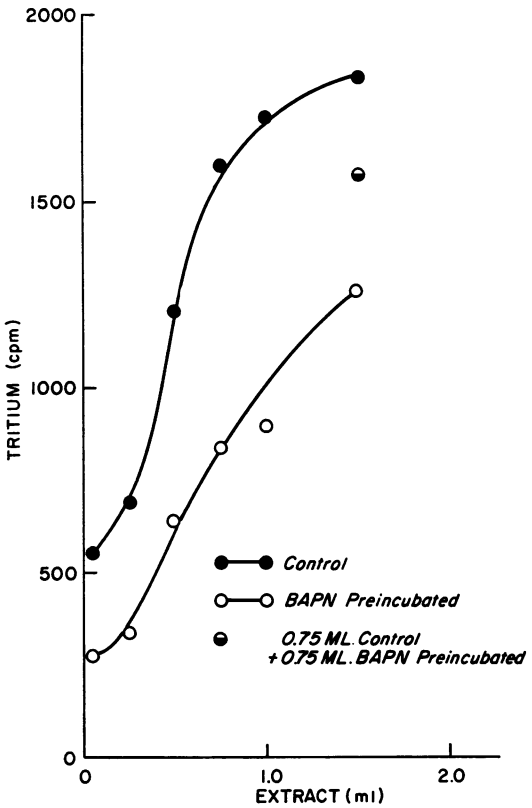

FIG. 5.—Enzymatically dependent release of tritium from lysine-6-^{3}H-labeled substrate. Partial inactivation of the enzyme after incubation with 50 μg/ml BAPN.

Other workers have drawn attention to the possible role of amine oxidases in the cross-linking of collagen and elastin.[17-19] The deamination of benzylamine[17, 18] by extracts from aorta is reduced in copper deficiency, a condition in which elastin cross-linking is also impaired. BAPN, an inhibitor of collagen and elastin cross-linking, can decrease the rate of amine metabolism by amine oxidases since it is also a substrate.[17, 19] However, several observations suggest that the enzyme described in this report differs from these amine oxidases. Many tissue amine oxidases are particulate, whereas the enzyme described here is present in the 105S fraction of the homogenate. Considerably higher levels of BAPN than those used here are required to demonstrate competition with amines for amine oxidases. Furthermore, BAPN does not inactivate amine oxidase[20] but does inactivate the enzyme carrying out the lysyl-allysyl conversion.

As yet, we have not been able to detect the formation of a desmosine cross-link following the enzyme-dependent lysyl-allysyl conversion. For this reason, we cannot determine if other enzymes are involved in the actual formation of the cross-links. Studies with purified enzyme preparations and more highly defined substrates should permit the resolution of this question.

Summary.—An enzyme is present in extracts of bone which converts peptide-bound lysine to peptide-bound α-aminoadipic-δ-semialdehyde, the intial step

500 ml H_2O. The data are expressed as ratios of ^{45}Ca inside the sac to that in the incubation medium.

Experiments on the mobilization of bone: Rats were fed the purified diet described earlier,[9] except that calcium was omitted. After 2–3 weeks on this low-calcium diet they were severely deficient, with serum calcium concentrations of 4.0–4.5 mg/100 ml. At this time the rats were given 2.5 μg of either vitamin D_3 or 25-OH D_3. At the indicated times thereafter, the rats were killed and the serum calcium concentration was determined by Webster's method.[13] The rise in serum calcium in rats on this diet reflects increased mobilization of bone.

Rickets prevention test in chicks: Day-old chicks obtained from the Sunny-Side Hatchery of Oregon, Wisconsin, were fed a rachitogenic diet[14] with graded levels of 25-OH D_3 or vitamin D_3 dispersed in the diet. Twenty chicks were used at each dosage level. After 21 days the chicks were sacrificed and the ash content of tibiae pooled from each dosage level was determined according to standard AOAC procedures.[14]

Results.—In many repeated "line test" assays of antirachitic activity with rats, the 25-hydroxycholecalciferol was always 1.3–1.5 times more active than crystalline vitamin D_3. Independent assays by the Wisconsin Alumni Research Foundation Laboratory gave the same result. Examples of such results are given in Table 1. The assays also show that intravenous administration of the 25-OH D_3 is just as effective as oral administration, thus demonstrating that passage through the intestinal mucosa is not essential to 25-OH D_3 activity.

The results shown in Table 2 demonstrate that 25-OH D_3 is extremely effective in initiating calcium transport in the duodenum of deficient rats. It is of great interest that even when administered intravenously, it initiates calcium transport much more rapidly than does vitamin D itself. A significant stimulation by 0.25 μg vitamin D_3 is observed only after six to ten hours, whereas 0.25 μg 25-OH D_3 produces a response within two to three hours after its administration. These

TABLE 1. *Comparative effectiveness of 25-hydroxycholecalciferol and vitamin D_3 in the cure of rickets in rats.*

Type of dosage	25-OH D_3 (IU/μg)	Vitamin D (IU/μg)
Oral (8)	58 ± 5*	40 ± 4*
Oral (7)	52 ± 3	38 ± 5
Intravenous (7)	56 ± 2	41 ± 3

The standard line test assay for vitamin D activity was carried out as described in *U.S. Pharmacopoeia*.[10] The figures in parentheses represent number of rats per group.
* Standard deviation.

TABLE 2. *Effect of intrajugular administration of 0.25 μg of 25-hydroxycholecalciferol or vitamin D_3 on calcium transport by everted intestinal sacs.*

Hours after administration	^{45}Ca Serosal/^{45}Ca Mucosal	
	Vitamin D_3	25-Hydroxycholecalciferol
Control	1.25 ± 0.05 (12)	
2		1.24 ± 0.13* (4)
3		1.74† ± 0.13 (4)
4	1.01 ± 0.12* (4)	1.66† ± 0.13 (4)
6	1.32 ± 0.10 (4)	2.6 ± 0.4 (4)
10	2.0 ± 0.3 (3)	2.3 ± 0.6 (4)

* Plus or minus the standard error of the mean. Numbers in parentheses show the numbers of animals in each group.
† $P < 0.01$ above control.

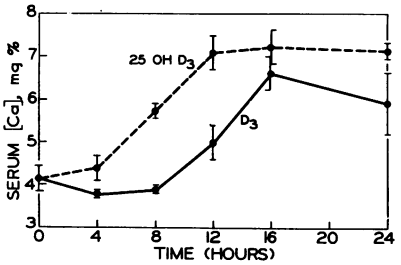

FIG. 1.—Serum calcium response to 2.5-μg intravenous doses of 25-hydroxycholecalciferol (- - -) and vitamin D_3 (—) in vitamin D-deficient rats on a low calcium diet. Each point is the mean (± SD) of duplicate assays of serial samples from three to five rats.

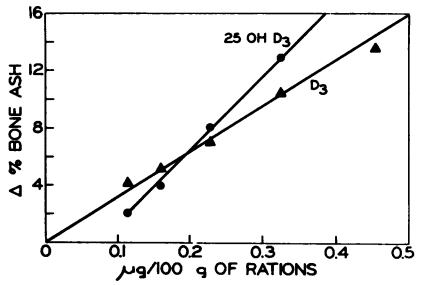

FIG. 2.—The increase in per cent bone ash over the controls for graded doses of 25-hydroxycholecalciferol (●) and vitamin D_3 (▲). Each point represents the pooled tibiae of 20 chicks.

TABLE 3. *Activity of 25-hydroxycholecalciferol as measured by the chick bone ash assay.*

Weight (μg)/100 gm of Rations		Body Weight during Assay Period (gm)		
D_3	25-OH D_3	Initial	Final	Bone ash
Oil Only		35	116	28.3*
0.113		35	142	32.4
0.159		35	145	33.4
0.225		35	151	35.3
0.319		35	166	38.7
0.450		35	152	41.8
	0.159	35	140	32.2
	0.225	35	151	36.3
	0.319	35	162	41.2

* Each assay is for the pooled tibiae from 20 chicks receiving each dose.

results suggest that at least a part of the lag in vitamin D action may result from the necessity for its conversion to 25-OH D_3 before it can act.

25-Hydroxycholecalciferol was also highly effective in eliciting a rise in serum calcium at the expense of bone in rats on a low calcium diet (Fig. 1). Again it is apparent that a significant elevation of serum calcium occurred 4–6 hours after administration of 2.5 μg of 25-OH D_3, whereas 8–12 hours was required for a similar response to vitamin D_3. The rise in serum calcium in response to vitamin D also can be shown with as little as 0.25 μg of 25-OH D_3.

In chicks the 25-OH D_3 is also more active than vitamin D_3. Exactly how much more active is not clear because at the higher dosage levels 25-OH D_3 was considerably more active than vitamin D_3, whereas at the two lowest dosage levels it appeared less active (Table 3 and Fig. 2). This may be due to instability of the 25-OH D_3 when dispersed in small concentrations in the diets. Other explanations are possible and additional investigations with chicks will be necessary to clarify this point.

Discussion.—It has been generally accepted that cholecalciferol or vitamin D_3 is the most potent antirachitic substance known. This report demonstrates that a metabolite of vitamin D_3, namely, 25-hydroxycholecalciferol, is even more potent than cholecalciferol. Its superior antirachitic potency is easily demonstrated in both rats and chicks, animals which differ markedly in their response to

in the cross-linking of collagen and elastin. The activity is inhibited by physiologically active levels of the lathyrogen β-aminopropionitrile.

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* A portion of this work has been presented elsewhere (abstract, S. R. Pinnell *et al.*, *Federation Proc.*, **27**, 588 (1968)).

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